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Overexpression of the Proteasome Subunits LMP2, LMP7, and MECL-1, But Not PA28α/β, Enhances the Presentation of an Immunodominant Lymphocytic Choriomeningitis Virus T Cell Epitope[1]

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The proteasome is a large protease complex that generates most of the peptide ligands of MHC class I molecules either in their final form or in the form of N-terminally extended precursors. Upon the stimulation of cells with IFN-γ, three constitutively expressed subunits of the 20S proteasome are replaced by the inducible subunits LMP2 (low-molecular mass polypeptide 2), LMP7, and MECL-1 (multicatalytic endopeptidase complex-like-1) to form so-called immunoproteasomes. We show in this study that overexpression of these three subunits in triple transfectants led to a marked enhancement in the H-2L^d-restricted presentation of the immunodominant nonameric epitope NP118, which is derived from the nucleoprotein (NP) of lymphocytic choriomeningitis virus. Overexpression of the α and β subunits of the IFN-γ-inducible proteasome regulator PA28, in contrast, did not have a comparable effect. In vitro, immunoproteasomes as compared with constitutive proteasomes generated higher amounts of 11- and 12-mer fragments containing the NP118 epitope. These are likely to be cytosolic precursors of NP118, as a proline anchor residue in the second position of NP118 may interfere with TAP-mediated transport of the nonameric epitope itself. In conclusion, we provide evidence that up-regulation of the three inducible subunits, LMP2, LMP7, and MECL-1, can result in a marked improvement of Ag presentation and that, depending on the epitope, PA28 and immunoproteasomes may differentially affect Ag processing. *The Journal of Immunology,* **2000, 165: 768–778.**

The peptide ligands of MHC class I molecules have to fulfill many criteria. To bind with high affinity to the peptide-binding groove of MHC class I molecules, they need to be of a restricted length of 8 or 9 aa and must contain certain amino acids as anchor residues (1). Moreover, they have to meet the substrate requirements of the TAP, which transports MHC ligands or their precursors from the cytosol into the lumen of the endoplasmic reticulum (ER)[4] (2). The optimal length of peptides for TAP-mediated transport lies between 8 and 12 aa, and both murine MHC and TAP transporters select for peptides with hydrophobic C termini. Moreover, peptides that contain a proline in positions 2 are inefficiently transported by human or mouse TAPs (2, 3). As the class I molecules H-2L^d in the mouse or HLA-B35 and HLA-B53 in the human require proline at position 2, this implies that their peptide ligands are probably produced in the cytoplasm as precursors of at least 10 aa in size. It has been shown that the C termini of MHC ligands are normally generated by proteases in the cytoplasm, while the final N termini can still be generated in the ER lumen by to date unidentified amino peptidases (4, 5).

Experiments employing inhibitors of the proteasome have meanwhile shown that it is the proteasome system (6) that generates most of the class I ligands and their precursors (7–10). This notion has been further corroborated by the discovery of two IFN-γ-inducible proteasome subunits named LMP2 and LMP7, which are encoded in the MHC class II locus. Upon their transcriptional induction, LMP2 and LMP7 replace two constitutive β-type subunits designated δ and MB-1, respectively, in newly assembled 20S proteasomes. The exchange of these proteasome subunits was shown to alter the cleavage specificity of the 20S proteasome in vitro. Replacement of the subunit δ by LMP2, for instance, dramatically reduced the proteasomal cleavages C terminal of glutamic acid, while the exchange of LMP7 for MB-1 was found to increase the cleavage C terminal of hydrophobic residues in one (11), but not in other studies (12–14). The in vivo effects of LMP2 and LMP7 were analyzed in gene-targeted mice and mutant cell lines. LMP7-deficient mice displayed a slight reduction of MHC class I molecules on lymphocytes and macrophages and a reduced ability to present the HY male Ag (15). The reduction in class I expression could be rescued by administration of exogenous peptides, indicating that the defect was due to a lack of appropriate peptide ligands. LMP2-deficient mice, in contrast, were not reduced in class I cell surface expression, but had a slightly reduced

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[4] Abbreviations used in this paper: ER, endoplasmic reticulum; IEF, isoelectric focusing; LCMV, lymphocytic choriomeningitis virus; LMP, low-molecular mass polypeptide; MALDI-MS, matrix-assisted laser desorption ionization-mass spectroscopy; MECL, multicatalytic endopeptidase complex-like; NP, nucleoprotein; TFA, trifluoroacetic acid; VV, vaccinia virus.

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level of $CD8^+$ T lymphocytes and generated fewer CTL precursors to an influenza NP epitope, while the response to Sendai virus was normal (16). LMP2 and LMP7 double-deficient lymphoblastoid cell lines showed no significant reduction in class I cell surface expression (17, 18) and no apparent defect in the class I-restricted presentation of several virus-derived T cell epitopes (19, 20). Only in a mouse cell line selectively deficient for LMP2 it could be shown that this subunit was required for presentation of two epitopes of influenza hemagglutinin (21). Taken together, the impact of LMP2 and LMP7 on Ag processing and presentation was evident for certain epitopes, but not very prominent for the bulk production of MHC class I ligands.

Recently, a third pair of subunit exchanges in the 20S proteasome has been discovered. The subunit MECL-1, which, in contrast to LMP2 and LMP7, is not encoded in the MHC, is inducible by IFN-γ and replaces a subunit that was designated Z in the human and MC14 or LMP9 in the mouse (22–24). The function of MECL-1 in Ag presentation has not been investigated to date, and cell lines or mice that are deficient for MECL-1 are not yet available. Interestingly, the incorporation of MECL-1 into the 20S proteasome is strictly dependent on the subunit LMP2 (25) and is accelerated in the presence of LMP7 (26), suggesting that these subunits are preferentially coincorporated, leading to the formation of so-called immunoproteasomes. As the constitutively expressed subunits δ, MB-1, and MC14 bear the three putative active centers of the proteasome, it appears that all three subunits have an IFN-γ-inducible homologue and are amenable to modulation during the immune response.

The 20S proteasome must be viewed as a proteolytic core complex that needs to associate with regulatory complexes that control the cleavage activity of the proteasome and the access of substrates to the lumen of the 20S proteasome. The PA700 complex (or 19S regulator) consists of at least 17 different subunits, and is thought to be required for the binding and unfolding of ubiquitinated proteins (6). The PA28 complex (or 11S regulator), in contrast, is constituted from two different subunits, PA28α and PA28β, which both are inducible by IFN-γ (27). They form rings of seven subunits with about equal stoichiometric amounts of α and β, which bind to the 20S proteasome (28–30). Both the PA28α and β subunits have been shown to enhance peptide hydrolysis by the 20S proteasome, but the respective functional contributions of α and β are still elusive (31–33). With respect to PA28 function, we proposed that it is involved in Ag processing, as overexpression of the PA28α subunit markedly enhanced the Ag presentation of two different virus-derived epitopes (34). Moreover, PA28 accelerated the processing of nonameric MHC ligands from polypeptide precursors by the 20S proteasome in vitro (35). Mechanistically, it was proposed that PA28 induces the proteasome to perform dual cleavages that immediately would liberate fragments of the appropriate size from polypeptides precursors rather than performing consecutive cleavages (35, 36). Although these experimental findings obtained in several models systems support the proposed role for PA28 in Ag processing, it remains to be shown whether all T cell epitopes similarly benefit from PA28 expression.

In this work, we aimed at dissecting the effects of IFN-γ on Ag processing by the proteasome system by the means of overexpressing either mouse PA28α and β or the subunits LMP2, LMP7, and MECL-1 in double and triple transfectants of mouse fibroblasts, respectively. As a model system for Ag presentation, we chose the infection of BALB/c-derived fibroblast lines with the lymphocytic choriomeningitis virus (LCMV). The cytotoxic immune response to LCMV is essential for elimination of the virus from infected mice. In BALB/c mice, this response is strongly dominated by CTLs specific for the H-$2L^d$-restricted nonameric epitope NP118 (37), which consists of residues 118–126 of the LCMV-NP and is generated in a proteasome-dependent manner (38). We report in this study that the presentation of NP118 is markedly enhanced through LMP2/LMP7/MECL-1 coexpression, and that the putative 11- and 12-mer precursors of this epitope are produced in vitro by immunoproteasomes much more efficiently as compared with constitutive proteasomes. Overexpression of PA28α/β, in contrast, had little effect on Ag presentation in this system. Taken together, our data demonstrate that the concerted expression of the IFN-γ-inducible proteasome subunits LMP2, LMP7, and MECL-1 may greatly enhance the efficiency of the intracellular T cell epitope production.

Materials and Methods

Mice and LCM virus

BALB/c mice (H-2^d) were purchased from the Institut für Labortierkunde, Tierspital Zürich (Zürich, Switzerland) and kept in a specific pathogen-free environment. LCMV-WE strain was originally obtained from F. Lehmann-Grube (Hamburg, Germany) (39). LCMV was propagated in the L929 fibroblast line, and viral stocks were kept at -70°C.

Synthetic peptides

The synthetic peptides were purchased from Echaz microcollections (Tübingen, Germany). The 25-mer encompassing LCMV-WE NP residues 108–132 KLKAKIMRTERPQASGVYMGNLTAQ contained the immunodominant 9-mer epitope (NP118–126), which is presented by H-$2L^d$.

Antibodies

For Western blot analysis, rabbit polyclonal Abs recognizing mouse LMP2 (26), LMP7 (Affinity, Mamhead, U.K.), and MECL1 (25) as well as the PA28α and PA28β subunits (40) were used. The secondary Ab was a goat anti-rabbit Ig HRP conjugate (Dako, Zug, Switzerland). For immunoprecipitation of the proteasome, a rabbit anti-mouse proteasome antiserum was used. The LCMV-NP was immunoprecipitated using the rat mAb VL4 (41). For surface staining of the H-$2L^d$, H-$2K^d$, and H-$2D^d$ molecules, the mAbs 28-14-81, 15-5-5S, and 19/191 were used and the LCMV-glycoprotein staining was performed with the mouse mAb KL 25 (42), followed by a FITC-conjugated sheep anti-mouse Ig (Silenus, Victoria, Australia). The intracellular staining of the LCMV-NP was performed using mAb VL4, followed by a PE-conjugated rabbit anti-rat IgG (Serotec, Oxford, U.K.).

Cell lines

The B8 clone was obtained from Dr. U. Koszinowski (Munich, Germany), and had been generated from the BALB/c-derived fibroblast cell line C4 by cotrancfection with the IE1/pp89 gene of the mouse CMV and a neomycin-resistance gene (13). B8 cells were grown in complete IMDM (10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin) supplemented with 250 μg/ml G418. The stimulation of B8 cells with IFN-γ was always done for 2 days with 20 U/ml mouse rIFN-γ (Life Technologies, Basel, Switzerland). Triple transfection of B8 cells with the BALB/c-derived LMP2, LMP7, and MECL1 cDNAs yielded the clones B27 M2 (25), B27 M6, and B27 M9. Transfection of the MECL-1 transfectant BME13 (25) with a LMP2 expression vector yielded the cell line B2 M1. Double transfection of B8 cells with the cDNA of mouse PA28α and β (40) generated the clones BP$\alpha\beta$2 and BP$\alpha\beta$13. These cells were cultured in selection medium consisting of complete IMDM supplemented with 2.5 μg/ml puromycin (Sigma, Buchs, Switzerland), 400 μg/ml hygromycin B (Roche, Basel, Switzerland), and 250 μg/ml G418 (Life Technologies). T2-L^d is the TAP-deficient human lymphoblastoid line T2 cell line (43) transfected with H-$2L^d$.

CTL lines and T cell hybridoma

CTL lines recognizing the LCMV-NP epitope NP118 were generated from spleen cell suspensions of LCMV memory mice that had been infected i.v. with 200 PFU LCMV-WE at least 1 mo before. Splenocytes were plated in complete IMDM supplemented with 5×10^{-5} M 2-ME and 10% rat Con A supernatant. Cells were restimulated every 7–10 days with NP118 peptide-loaded and irradiated (80 Gy) T2-L^d cells at a ratio of CTL to APC of 5:1. Loading with synthetic peptides was for 1 h at room temperature with 10^{-7} M of peptide. Generally, the CTL line was found to be exclusively

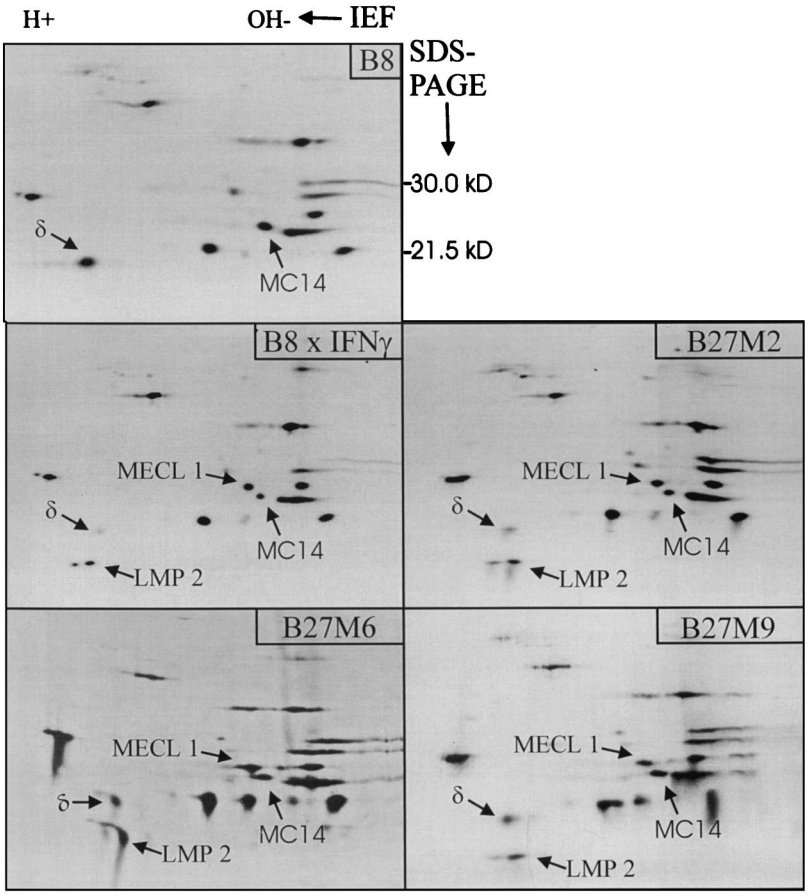

FIGURE 1. Two-dimensional IEF/PAGE analysis of proteasomes immunoprecipitated from three different LMP2/LMP7/MECL-1 triple transfectants (B27 M2, B27 M6, and B27 M9) as well as the parental B8 line grown in the presence and absence of IFN-γ. The cells were labeled with [^{35}S]methionine/cysteine for 4 h and then grown for 5 h in chase medium to allow full maturation of 20S proteasomes. The positions of the housekeeping subunits δ and MC14 and the IFN-γ-inducible subunits LMP2 and MECL1 are indicated on the autoradiographies.

specific for NP118/H-2L^d after three rounds of restimulation. For the ex vivo CTL assay, mice were infected i.v. with 200 PFU LCMV-WE, and after 8 days, the mice were sacrificed and a spleen cell suspension was prepared for immediate use as effector cells in a standard chromium release assay. The NP-118-specific hybridoma clone HNP-118 resulted from a fusion of an LCMV NP118-specific CTL line with the TCR-deficient lymphoma BWZ36.1 (44) expressing CD8α and a *lacZ* reporter construct under the control of the IL-2 promotor/enhancer (38). These cells were grown in complete IMDM/hypoxanthine, aminopterin, thymidine (HAT; Sigma) supplemented with 0.5 mg/ml hygromycin B.

Generation of rVV-NP118

We cloned the synthetic DNA sequence encoding the nonameric NP118–126 epitope plus one additional methionine at the N terminus (MRPQASGVYM) into the vaccinia vector pSC11.3OR2 (45). The plasmid DNA was used to cotransfect thymidine kinase-negative 143B fibroblasts (American Type Culture Collection (ATCC), Manassas, VA) after their infection with wild-type vaccinia virus. Successful homologous recombination of the vaccinia vector with the vaccinia virus was assessed by selection for 5-bromo-2′-deoxyuridine-resistant, β-galactosidase-expressing virus plaques. Plaques were selected and replaqued three times until one isolated plaque was chosen for amplification on BS-C-40 (ATCC) cells.

Purification of 20S proteasome

The lysis, purification, and quantitation of the 20S proteasome from B8 cells were performed exactly as described (13).

Metabolic labeling and immunoprecipitation

Cells grown to confluence were starved in cysteine/methionine-free RPMI 1640, 10% dialyzed FCS for 1 h at 37°C and labeled with 0.2 mCi/ml Tran^{35}S label (ICN, Eschwege, Germany) for the indicated time. Labeling medium was removed and cells were washed with PBS, harvested, and lysed for 30 min at 4°C in 50 mM Tris/HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.75 μM aprotinin, 10 μM leupeptin, 2.8 μM pepstatin, and 0.85 mM PMSF. The postnuclear lysates were counted for ^{35}S incorporation, and equal aliquots were used for immunoprecipitation. The lysate was precleared for 1 h at 4°C with preimmune serum coupled to protein G-Sepharose CL-4B (Pharmacia, Uppsala, Sweden), followed by immunoprecipitation with the indicated Ab bound to protein G-Sepharose for 3 h at 4°C. The precipitates were washed with PBS, pH 7.5, 0.1% Triton X-100, and separated on IEF and/or SDS-PAGE (performed as described in Ref. 23). The proteins were visualized by autoradiography on x-ray films or by using a BAS 1500 radioimager (Fuji, Tokyo, Japan).

Western blot analysis

Cells were lysed in 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 0.75 μM aprotinin, 10 μM leupeptin, 2.8 μM pepstatin, and 0.85 mM PMSF for 30 min at 4°C. The postnuclear supernatant was quantified by OD, and aliquots of 130 μg protein were applied to a 12% SDS-PAGE. The proteins were blotted onto nitrocellulose membrane (Schleicher und Schuell, Dassel, Germany), blocked with PBS/10% horse serum/5% (w/v) low fat dry milk/0.4% Tween-20, and agitated overnight at 4°C with the indicated Ab in PBS/2% low fat dry milk/0.1% Tween-20. The blots were washed and incubated for 1 h with the HRP-conjugated secondary Ab. After extensive washing with PBS/0.2% Tween-20, proteins were visualized on x-ray films by enhanced chemiluminescence.

Proteasomal fragmentation of polypeptides and analysis of peptide products

A total of 80 μg of a synthetic 25-mer peptide derived from the sequence of the LCMV-NP (NP108–132) was incubated with 4 μg purified 20S proteasome in a total volume of 1200 μl of digestion buffer (30 mM Tris/HCl, pH 7.5, 10 mM KCl, 2 mM DTT) at 37°C. At indicated time points, aliquots of 300 μl were removed and frozen to stop the reaction. These cleavage products were separated on a μRPC C2/C18 SC 2.1/10 reverse-phase column using a SMART System (Pharmacia). Eluent A, 0.1% trifluoroacetic acid (TFA); eluent B, 70% acetonitrile + 0.1% TFA. Gradient 10–30% B in 55 min, flow rate 100 μl/min. Peak fractions were collected, vacuum dried, and resolved in 60% acetonitrile, 0.1% TFA. For the identification of peptides, the samples were coprecipitated with a matrix of α-cyano-4-hydroxycinnamic acid in acetone and analyzed by MALDI-MS

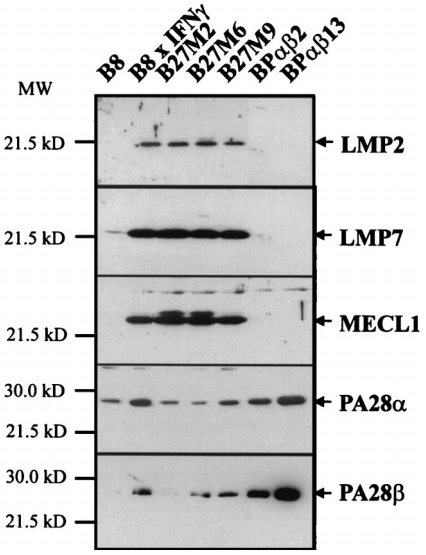

FIGURE 2. Western blot analysis of the expression of LMP2, LMP7, MECL-1, PA28α, and PA28β. Total lysates of untreated or IFN-γ-stimulated B8 cells; the LMP2/LMP7/MECL-1 triple transfectants B27 M2, B27 M6, and B27 M9; as well as the PA28α/β transfectants BP$\alpha\beta$2 and BP$\alpha\beta$13 were blotted and probed with polyclonal antisera of the respective specificities. The relative molecular masses (indicated on the *left*) corresponded well to the predicted M_r of the respective murine proteins, as indicated.

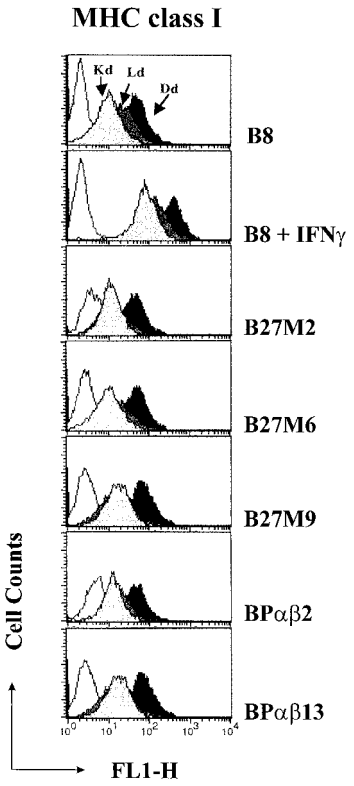

FIGURE 3. Flow-cytometric analysis of MHC class I cell surface expression of untreated or IFN-γ-stimulated B8 cells; the LMP2/LMP7/MECL-1 triple transfectants B27 M2, B27 M6, and B27 M9; as well as the PA28α/β transfectants BP$\alpha\beta$2 and BP$\alpha\beta$13. Cells were stained for the H-2K^d, H-2L^d, and H-2D^d class I molecules, as indicated, followed by a FITC-conjugated sheep anti-mouse Ig secondary Ab (filled curves). The open curves are stainings with the secondary Ab alone.

(VG-TofSpec; Fison Instruments, Manchester, U.K.). For microsequence analyses of the HPLC-separated peptide samples, a Procise protein sequencer system (Applied Biosystems, Foster City, CA) was used.

Cytolytic assays

Target cells (B8 fibroblasts and transfecants thereof) were infected either for 24 h with LCMV-WE at a multiplicity of infection of 0.01 or for 1 h with rVV-NP118 with a multiplicity of infection of 3. One million cells were labeled in 200 μl with 100 μCi $Na_2{}^{51}CrO_4$ for 1 h at 37°C before washing and incubation of 10^4 cells/well in a 96-well plate with effector CTL at indicated E:T ratios at 37°C. Chromium release was measured after 4 h in 70 μl of culture supernatant. For determining the spontaneous release, the supernatant of target cells without adding effector CTL was measured. The total release was determined by adding 0.1% Nonidet P-40 (final concentration) to labeled targets. Specific lysis was calculated as (experimental release − spontaneous release)/(total release − spontaneous release) × 100%. All samples were measured in triplicates.

lacZ *assay*

The LCMV-NP118-specific hybridoma clone HNP-118 was cocultured overnight with 5×10^4 LCMV-infected target cells in 96-well plates at an E:T ratio of 10:1, 5:1, and 1:1. The cultures were washed once with PBS and lysed by addition of 100 μl of Z buffer (0.15 mM chlorophenol red β-galactoside (CPRG; Roche), 100 mM 2-ME, 9 mM $MgCl_2$, 0.125% Nonidet P-40 in PBS). After 4 h of incubation at 37°C, the absorbance at 570 nm (reference wavelength at 620 nm) was read using a SpectraFluor Plus plate reader (Tecan, Gröding, Austria).

Viability assay

A total of 20 μl Cell Titer 96 AQueous One Solution Reagent was added to 5×10^4 cells in 100 μl medium in 96-well plates. The plates were incubated for 1 h at 37°C before the absorbance was recorded at 450 nm using the SpectraFluor Plus plate reader.

Flow cytometry

For surface staining, aliquots of 4×10^5 cells in PBS + 2% FCS were incubated for 15 min in a round-bottom 96-well plate on ice with the indicated Ab, washed three times, and subsequently stained by FITC-conjugated secondary Ab. The LCMV-NP was detected by intracellular staining. In brief, aliquots of LCMV-infected cells were permeabilized with 0.1% saponin (Sigma) in PBS + 2% FCS for 10 min, and the staining was performed in this buffer until completion. The NP was stained for 30 min with the VL4 Ab, washed twice, and subsequently stained by PE-conjugated Ab for an additional 30 min. After two washing steps, the staining was analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Results

The stimulation of cells with IFN-γ was estimated to induce up to 400 genes, many of which can be expected to influence the MHC class I-restricted Ag presentation pathway (46). In this work, we wanted to separately investigate how an incorporation of the subunits LMP2, LMP7, and MECL-1 into the proteasome as it occurs subsequent to IFN-γ stimulation would affect Ag processing and compare it with the effect of PA28 up-regulation. To this end, we created transfectants overexpressing either the α and β subunits of PA28 or the three IFN-γ-inducible subunits of the immunoproteasome.

Characterization of transfectants expressing LMP2/LMP7/MECL-1 and PA28α/β

For the generation of stable transfectants, we chose the mouse fibroblast line B8, which endogenously expresses little LMP2, LMP7, and MECL-1. Expression constructs encoding these three subunits were transfected, and after isolation of drug-resistant cells, three different clones named B27 M2, B27 M6, and B27 M9, which according to Northern analysis strongly overexpressed the respective mRNAs (data not shown), were chosen for further characterization. The 20S proteasomes of these transfectants and, for

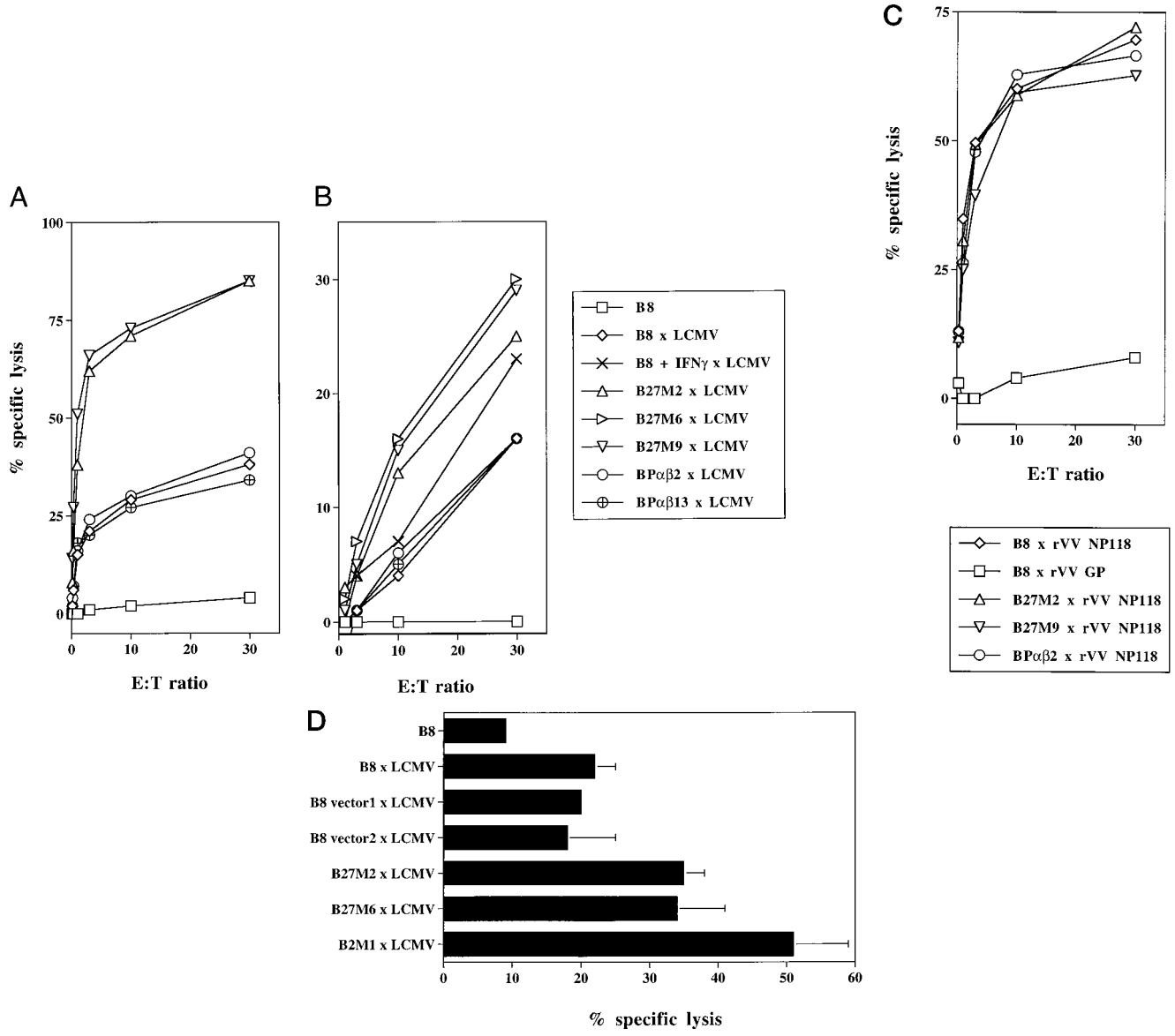

FIGURE 4. Comparison of the H-2L^d-restricted presentation of the LCMV-NP118 epitope by LCMV or rVV-NP118-infected cell lines transfected with LMP2/LMP7/MECL-1 or PA28α/β in cytolytic assays. *A*, Cytolytic assay with NP118/H-2L^d-specific CTLs as effectors obtained from LCMV-infected BALB/c mice by repeated in vitro restimulation with the NP118 peptide. Target cells were IFN-γ-stimulated or unstimulated B8 cells; the LMP2/LMP7/MECL-1 triple transfectants B27 M2, B27 M6, and B27 M9; as well as the PA28α/β transfectants BP$\alpha\beta$2 and BP$\alpha\beta$13. *B*, Ex vivo cytolytic assay using spleen cells from BALB/c mice 8 days after infection with LCMV as effectors and targets, as in *A*. The ex vivo cytotoxic response in mice of the H-2^d haplotype to LCMV is known to be directed predominantly to the NP118 epitope. *C*, NP118-specific cytolysis of transfectants after infection with rVV-NP118 encoding the NP118 epitope as a minigene. As negative control, B8 cells were infected with rVV-glycoprotein encoding the LCMV glycoprotein. *D*, Cytolytic assay comparing NP118 presentation in LCMV-infected B8 recipient cells and clones transfected with the empty expression vector (B8 vector 1, B8 vector 2), or with expression constructs encoding LMP2/LMP7/MECL-1 (B27 M2, B27 M6) or LMP2/MECL-1 (B2 M1) at an E:T ratio of 10. Data represent the means of three replicate cultures; representative results from three independent experiments are shown.

comparison, of IFN-γ-stimulated and unstimulated B8 cells were immunoprecipitated under stringent conditions, and the composition of proteasome subunits was analyzed on two-dimensional IEF/PAGE. As shown on autoradiographies in Fig. 1, the three triple transfectants expressed LMP2 and MECL-1 in amounts that were comparable with those of IFN-γ-stimulated B8 cells, while in untreated B8 cells these subunits were not detectable. The constitutively expressed proteasome subunits δ and MC14 were replaced to a large extent by LMP2 and MECL-1, respectively, in the triple transfectants and in IFN-γ-treated cells in accordance with previous reports (22–24). Unfortunately, the LMP7 subunit has a basic isoelectric point, and hence migrates out of the IEF gel of our two-dimensional electrophoretic system. Therefore, we examined the expression level of LMP7 as well as of LMP2 and MECL-1 in Western analysis. As shown in Fig. 2, the inducible subunits are found in the triple transfectants in similar amounts as in IFN-γ-treated B8 cells, while in unstimulated B8 cells neither LMP2 nor MECL-1 and only little LMP7 were detected. Thus, the Western results are congruent with the immunoprecipitation analysis (Fig. 1) and a previous examination of purified 20S proteasomes from the triple transfectant B27 M2 and IFN-γ-treated and untreated B8 cells on Coomassie-stained nonequilibrium pH-gradient gel electrophoresis (NEPHGE)/PAGE two-dimensional gels (13, 23, 25). From these gels, it was apparent that the exchange of LMP7 for

MB-1 was complete in the B27 M2 transfectant and B8 cells after treatment with IFN-γ for 3 days.

To compare the joint effects of LMP2, LMP7, and MECL-1 on Ag presentation with that of PA28, we have generated double transfectants overexpressing both the PA28α and β subunits. Two clones designated BPαβ2 and BPαβ13 were chosen for further characterization, as they strongly expressed both mRNAs in Northern analysis (not shown). Western analysis revealed that the PA28α expression in these clones was comparable with IFN-γ-treated B8 cells and exceeded that of the low basal expression in B8 recipient cells by a factor of about 3 (Fig. 2). The PA28β protein was barely detectable in B8 cells, but prominently expressed in the BPαβ2 and BPαβ13 transfectants to an amount that was equal or even higher than found in IFN-γ-treated cells.

The overexpression of LMP2, LMP7, and MECL-1 or PA28α and β does not alter class I cell surface expression

A potential function that is discussed both for the inducible subunits of the immunoproteasome and for PA28 is an enhancement in the production of appropriate peptide ligands for class I presentation. As MHC ligands are required for the stabilization and cell surface transport of class I molecules, we investigated whether their surface expression would be altered by LMP2/LMP7/MECL-1 or PA28α/β overexpression. The B8 fibroblast line was originally derived from a BALB/c mouse, and hence expresses the MHC class I molecules H-2L^d, H-2D^d, and H-2K^d. A flow-cytometric analysis revealed that neither the joint overexpression of LMP2, LMP7, and MECL-1 nor of PA28α and β had a significant effect on the surface expression of these class I molecules (Fig. 3). Only the treatment of B8 cells with IFN-γ resulted in an about 10-fold increase in class I surface expression. This result indicates that if there is a change in Ag processing in the transfectants, this cannot be attributed to a general alteration of MHC class I cell surface expression.

The coexpression of LMP2, LMP7, and MECL-1 enhanced the Ag presentation of an LCMV-NP epitope

The cytotoxic immune response to LCMV in mice of the H-2^d haplotype is predominantly directed against a H-2L^d-restricted peptide epitope called NP118, which is constituted from the LCMV-NP residues 118–126 (37). We decided to compare the presentation of the NP118 epitope of LCMV-infected B8 cells with that of LMP2/LMP7/MECL-1 and PA28α/β transfectants. The cells were LCMV infected and used after 24 h as targets in a cytolytic chromium release assay employing NP118/H-2L^d-specific CTLs as effectors. Remarkably, the LMP2/LMP7/MECL-1 triple transfectants showed a strong enhancement of cytolysis by NP118-specific T cells when compared with parental B8 cells. As can be seen in Fig. 4*A*, two to three times more LMP2/LMP7/MECL-1-transfected cells compared with B8 cells were lysed by the same effectors in several independent experiments, and this difference could not be overcome by increasing the amounts of effector cells to higher E:T ratios. Interestingly, the PA28α/β double transfectants were lysed by NP118-specific CTLs to a similar extent as untransfected B8 cells. The latter finding differs from previous results obtained with PA28α single transfectants of B8 using CTLs specific for the influenza NP or the mouse CMV immediate early protein pp89 (34), suggesting that the effects of PA28 may not promote Ag presentation in different systems to the same degree.

To confirm and extend our findings in another experimental setting, we repeated this test as an ex vivo cytolytic assay using the same cells as well as IFN-γ-stimulated B8 cells as targets and freshly isolated CTLs from LCMV-infected mice as effectors. As

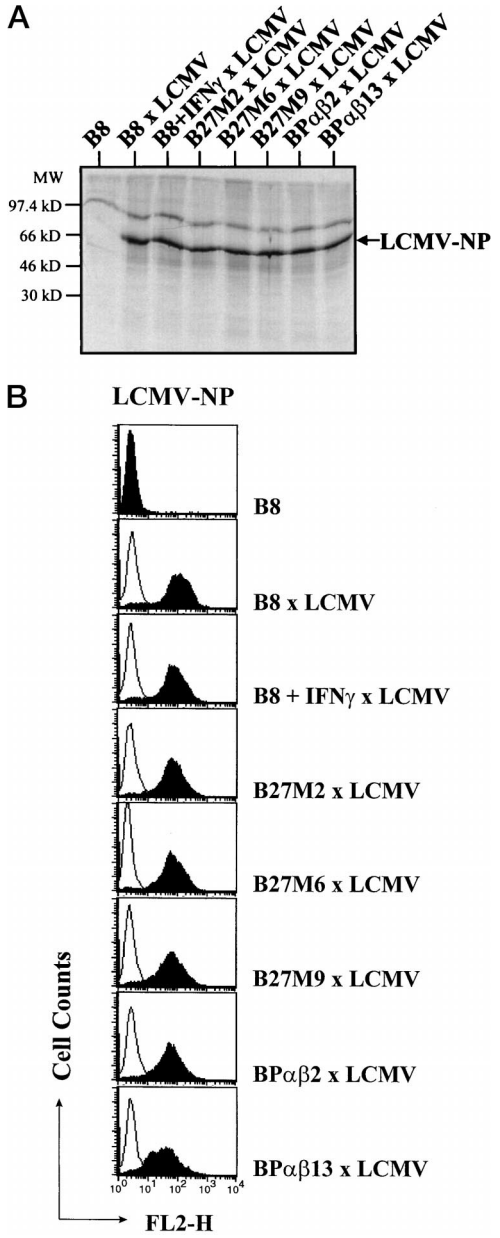

FIGURE 5. Quantification of LCMV-NP in different infected target cells used for cytolytic assay. *A*, Immunoprecipitation of the LCMV-NP from LCMV-infected target cells as prepared for the ex vivo cytolytic assay shown in Fig. 4*B*. Cells were labeled with [^{35}S]methionine/cysteine for 2 h, and LCMV-NP was immunoprecipitated with mAb VL4. Shown is an autoradiography of 15% SDS-PAGE; the position of M_r markers is indicated on the *left*, and the position of LCMV-NP on the *right*. *B*, Flow-cytometric quantitation of NP expression in LCMV-infected target cells 24 h after they were used for ex vivo cytolytic assay. Infected cells were stained intracellularly with the LCMV-NP-specific Ab VL4, followed by a PE-conjugated secondary Ab (filled curves); the open curves represent control stainings with the secondary Ab alone.

it has been shown that in a primary H-2^d-restricted immune response to LCMV no epitopes other than NP118 significantly contribute to cytolysis (47), it is safe to assume that the results obtained in a polyclonal ex vivo response reflect the recognition of NP118. Also, in this set up, the LCMV-infected LMP2/LMP7/MECL-1 triple transfectants were recognized much better than B8 cells or the PA28α/β transfectants (Fig. 4*B*). Moreover, we found that the treatment of B8 cells with IFN-γ enhanced the cytolysis to

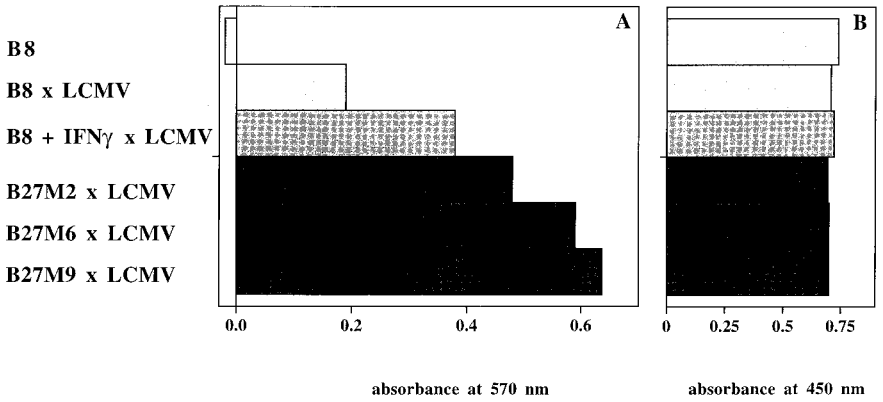

FIGURE 6. *A*, The stimulation of a LCMV-NP118-specific hybridoma through various LCMV-infected cells as monitored in a *lacZ* chromogenic assay. Stimulator cells were untreated or IFN-γ-stimulated B8 cells and the LMP2/LMP7/MECL-1 triple transfectants B27 M2, B27 M6, and B27 M9. Stimulator cells were infected 24 h before they were incubated with hybridomas at a hybridoma to stimulator cell ratio of 5:1. The absorption of the generated dye at 570 nm is plotted for each stimulator cell. All LCMV-infected stimulator cells expressed comparable levels of LCMV-NP, as determined by immunoprecipitation (not shown). One representative experiment of three is shown; data represent the mean of triplicate values. *B*, Viability staining to normalize for the number and viability of stimulator cells. The absorption at 450 nm of a dye produced in viable stimulator cells was measured.

a similar degree as the overexpression of LMP2, LMP7, and MECL-1, suggesting that immunoproteasomes contribute considerably to this enhancement. To confirm that the elevated NP118 presentation in LMP2/LMP7/MECL-1 triple transfectants was due to an enhancement in epitope generation and not to unrelated defects in the control cell lines, we generated the vaccinia recombinant rVV-NP118 encoding the nonameric NP118 epitope as a minigene. Infection with rVV-NP118 led to a high lysis by NP118-specific CTLs, which was equal for the B8 recipient and the transfectants, indicating that the enhanced NP118 presentation in LCMV-infected LMP2/LMP7/MECL-1 triple transfectants was due to a better efficiency in Ag processing (Fig. 4*C*). As LMP2 and MECL-1 on the one hand and LMP7 on the other hand can incorporate into the 20S proteasome independently, we decided to investigate how an LCMV-infected LMP2/MECL-1 double transfectant of B8 would present the NP118 epitope. As shown in Fig. 4*D*, also in the LMP2/MECL-1 double transfectant B2 M1, the NP118 epitope was presented at a higher level, suggesting that expression of LMP2 and MECL-1 is sufficient to achieve this enhancement. To ensure that the observed effects were not due to clonal variation or transfection artefacts, we tested two B8 clones that were transfected with the empty expression vector in the same experiment, and they, as expected, presented the NP118 epitope at the same level as the B8 recipient cells (Fig. 4*D*).

To exclude that the increase in presentation of NP118 in the triple transfectants was due to higher LCMV replication and NP synthesis in these cells, we immunoprecipitated the NP from the same batch of cells that were used as target cells in the ex vivo cytolytic assays (Fig. 4*B*). As shown in Fig. 5*A*, the NP content was not significantly different in the LMP2/LMP7/MECL-1 triple transfectants, the PA28α/β double transfectants, or B8 cells in the presence or absence of IFN-γ. This result was further confirmed by flow-cytometric analysis, which revealed identical levels of LCMV-NP expression in LCMV-infected B8 cells and the transfectants (Fig. 5*B*). Thus, we conclude that the improved recognition of LMP2/LMP7/MECL-1 triple transfectants must be due to a greater efficiency in the intracellular generation of the NP118 epitope rather than a higher level of NP synthesis in these cells.

Recently, we have produced a T cell hybridoma designated HNP-118, which is specific for the NP118 epitope presented on H-2L^d and which contains a β-galactosidase reporter gene under the IL-2 promotor/enhancer. The recognition of H-2L^d/NP118 and activation of the hybridoma can thus be quantitated in a chromogenic assay of β-galactosidase activity. Also, in this assay, the LMP2/LMP7/MECL-1 triple transfectants were recognized much better compared with parental B8 cells (Fig. 6*A*), although the cell number and viability (Fig. 6*B*) as well as LCMV-NP expression (data not shown) were identical among stimulators.

Taken together, it appears from three independent experimental systems that the incorporation of LMP2, LMP7, and MECL-1 into the proteasome in vivo markedly enhanced the production of NP118 epitopes and/or their precursors. As the incorporation of the IFN-γ-inducible subunits is believed to alter the peptide processing of the 20S proteasome, we decided to investigate in vitro whether evidence for altered processing can be obtained in this model and whether these changes are consistent with the observed in vivo effects.

Immunoproteasomes greatly favor the in vitro production of putative cytosolic precursors of the NP118 epitope

We purified 20S proteasomes both from B8 cells and from the triple transfectant B27 M2 and monitored the fragmentation of a synthetic 25-mer polypeptide from the sequence of LCMV-NP that contained the nonameric NP118 epitope. After several time periods, aliquots were removed from the proteolytic reaction and the fragments were separated by reversed phase HPLC (Fig. 7) and identified by MALDI-MS and automated Edman degradation. It is apparent that the same kind of fragments were produced by constitutive proteasomes from B8 cells and immunoproteasomes isolated from B27 M2 cells. However, although the kinetics of 25-mer consumption was comparable, the quantity of the respective peptide fragments produced differed markedly between the two proteasome populations. These fragments accumulated over the time of the digest, indicating that these differences in fragment quantity were already apparent at early time points of the cleavage reaction when the 25-mer substrate was still in excess.

To assign and quantify fragments that could be especially relevant for the generation of the NP118 epitope, Edman degradation was performed. Remarkably, the amounts of 11-and 12-mer fragments that contained the NP118 epitope (Fig. 8) were produced by immunoproteasomes in about 6- and 3-fold higher amounts, respectively. A 10-mer containing NP118 was produced in double amounts by constitutive proteasomes, but it was not a prominent fragment in either of the reactions. Also, the NP118 nonamer

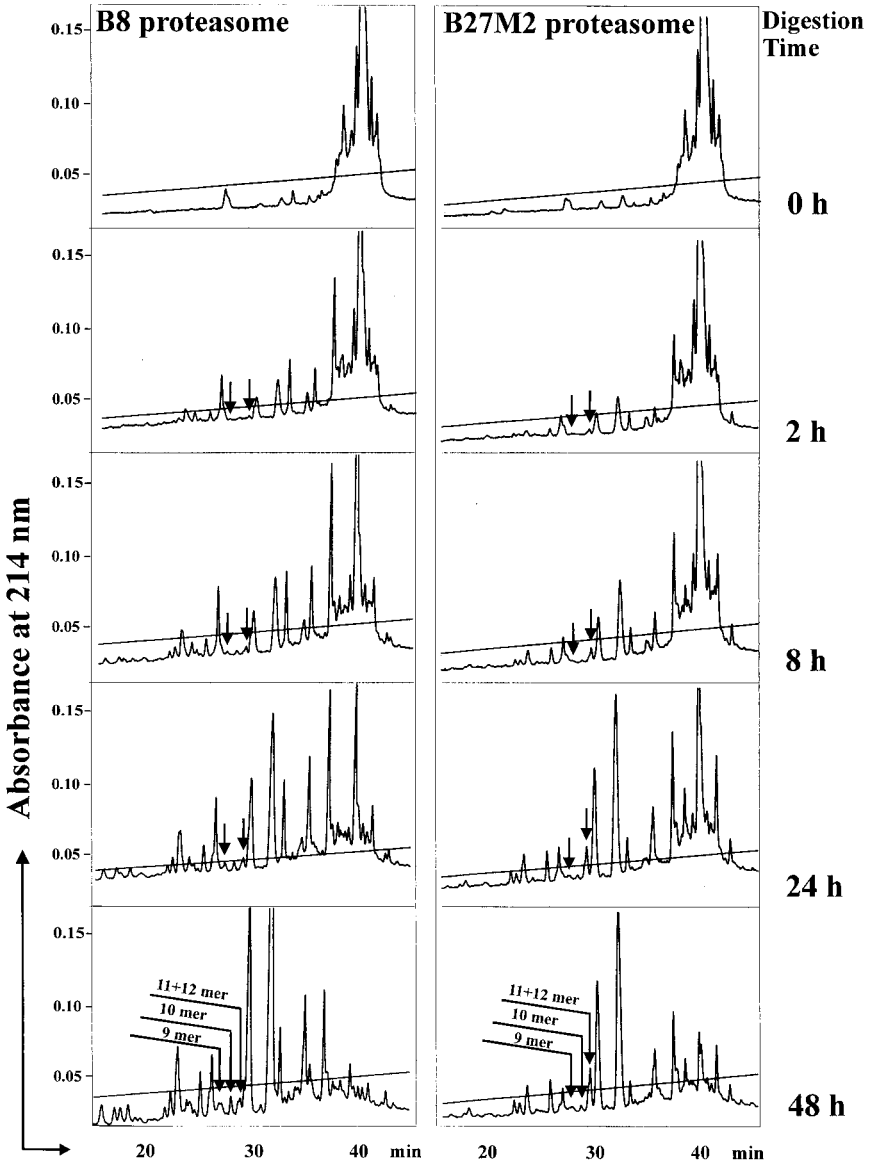

FIGURE 7. HPLC profile of fragments generated by purified 20S proteasomes from a synthetic 25-mer polypeptide in vitro. The 20S proteasomes were purified from B8 cells or the LMP2/LMP7/MECL-1 triple transfectant B27 M2. The sequence of the 25-mer polypeptide spans residues 108–132 of the NP of LCMV-WE and contains the immunodominant epitope NP118 (residues 118–126). At indicated time points, aliquots were removed from the peptidolytic reaction and analyzed. The arrows indicate the retention time of the 9-mer peptide NP118, the 10-mer peptide (residues 117–126), and the peak containing the 11 and 12 mer spanning residues 116–126 and 115–126, respectively.

epitope itself was not a prominent peptide product, and quantitation by Edman degradation required that the HPLC fraction containing NP118 (as identified by MALDI-MS) was pooled from several runs. This analysis revealed that the NP118 nonamer was produced by constitutive proteasomes to 1.5-fold greater amounts than by immunoproteasomes. Given that the putative 11- and 12-mer precursors, but not the NP118 nonamer, meet the criteria for an efficient transport by TAP transporters (see *Discussion*), these in vitro results are consistent with an increased presentation of the NP118 epitope in cell lines overexpressing LMP2, LMP7, and MECL-1.

Discussion

The discovery of the genes for the IFN-γ-inducible proteasome subunits LMP2 and LMP7 in the MHC class II locus 10 years ago had raised great expectations with respect to the function of these subunits in MHC class I-restricted Ag presentation. The phenotypes of cell lines or mice deficient for LMP2 and/or LMP7, however, were subtle and in many systems not apparent. In previous studies, we and others have tried to characterize the impact of LMP2 and LMP7 on Ag presentation by overexpressing these subunits, thus imitating their induction by IFN-γ (13, 48). Despite strong overexpression of LMP2, there was only little LMP2 protein incorporated into the 20S proteasome compared with IFN-γ-stimulated cells. The reason for this is now known: the third IFN-γ-inducible exchange of MC14 (or Z) by MECL-1 is required for incorporation of LMP2 (25, 26), which explains why LMP2 could only be incorporated efficiently into the proteasome in rare clones that displayed an endogenous up-regulation of MECL-1 expression (49). Accordingly, the coexpression of LMP2, LMP7, and MECL-1 yielded a good incorporation of all three subunits into the 20S proteasome in transfectants similar to immunoproteasomes from IFN-γ-treated cells (Fig. 1). This was the prerequisite to study their effect on Ag presentation by means of overexpression.

A key finding of our study is that the presentation of the immunodominant NP118 epitope by H-2L^d is markedly enhanced in transfectants overexpressing LMP2, LMP7, and MECL-1. At first sight, our in vitro results seemed to be at odds with this finding, as the nonameric NP118 epitope was a minor product in proteasome digests and was even generated by constitutive proteasomes in 1.5-fold greater amounts compared with immunoproteasomes. However, the NP118 epitope bears a proline as an anchor residue

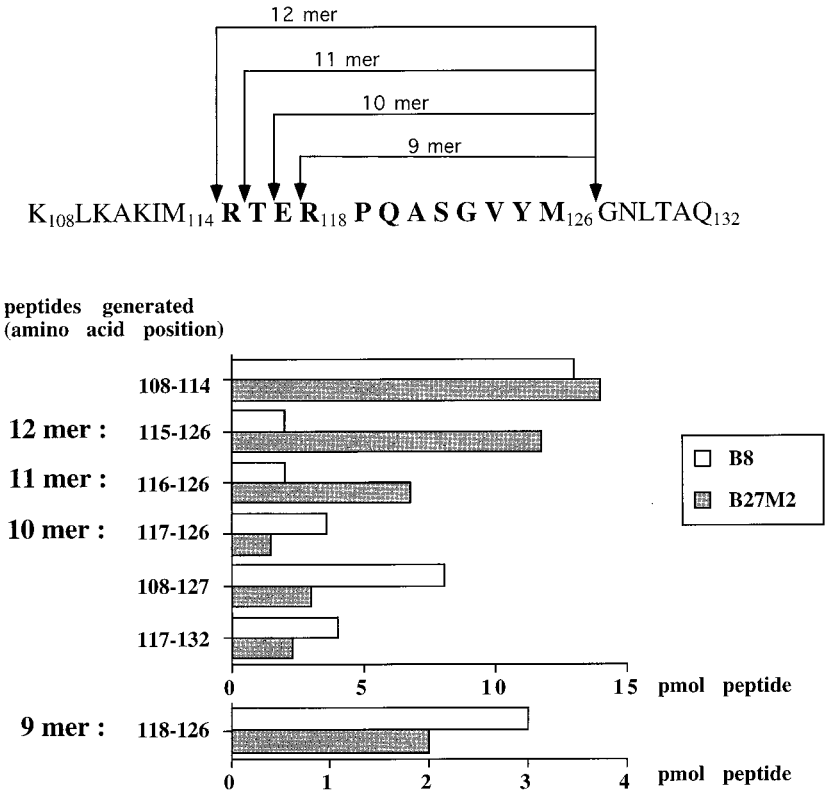

FIGURE 8. Quantitation of selected fragments produced from the LCMV-NP 25-mer polypeptide by 20S proteasomes purified from B8 cells and B27 M2 cells in vitro. The sequence of the 25- and 9-mer NP118 as well as putative precursor fragments are indicated above. The quantitation was performed by automated Edman degradation of peptide fragments that were obtained after 48 h of in vitro digest and fractionation by HPLC, as shown in Fig. 7. Quantitation of the NP118 nonamer by Edman degradation (*bottom*) could not be accomplished from the same single run as the other fragments, but required pooling of the NP118-containing fractions from several HPLC runs separating in total a 12-fold greater amount of peptide product.

for binding to the H-2L^d class I molecule in position 2. Mouse and human TAP transporters hardly transport peptides with proline in position 2 from the cytosol into the ER lumen, suggesting that N-terminally elongated precursors need to be formed and transported (3, 2). Interestingly, two potential cytosolic precursors of this epitope (the 11 or 12 mer in the scheme of Fig. 8) were produced in 3- and 6-fold greater amounts in vitro if LMP2, LMP7, and MECL-1 were incorporated into the 20S proteasome. The 11 and 12 mer are good candidates for precursors of the NP118 epitope because they have the appropriate C terminus, which normally cannot be generated by peptidases other than the proteasome (5) and because they are not too long to be efficiently transported by TAP transporters.

The NP118 epitope (shown in Fig. 8) is directly preceded by a glutamic acid residue at position 117. Proteasomal cleavage events at the C terminus of glutamic acid have been unanimously shown to be down-regulated by replacement of the constitutive subunit δ through LMP2 when proteasome activity was measured with help of fluorogenic peptides (11–14) or polypeptides (36). This finding would be consistent with our in vitro result that the generation of the NP118 9 mer, which requires the cleavage C terminal of Glu^{117}, is generated less efficiently by LMP2-containing immunoproteasomes as compared with constitutive proteasomes. The generation of the 11- and 12-mer precursors, in contrast, may have been enhanced by suppressing cleavages at Glu^{117}. If the NP118 nonamer cannot be efficiently conveyed by the TAP transporter, then the suppression of the cleavage after Glu^{117} would favor the generation of the required precursors, and thus enhance Ag presentation as we have observed. The suppression of cleavages C terminal of glutamic acids may also explain earlier results by Sibille et al. (21), who noted that LMP2 was required for the presentation of two influenza hemagglutinin epitopes, HA11 (FEANGNLI) and HA8 (IEGGWTGMI), both of which contain glutamic acid residues within their sequence.

Another interesting residue in the NP118 epitope is the proline in the second position serving as an anchor residue for H-2L^d. A recent analysis of how the proteasome may process polypeptides containing proline residues suggested that proline within an epitope sequence may preserve the epitope from destruction by proteasome-mediated internal cleavages (50). This proposal would fit nicely with the fact that proline is an anchor residue for several human and murine MHC class I molecules. It has been shown by inhibitor studies (51, 52) and the statistical analysis of proteasomal cleavage products (53) that it is not only the P1 residue that defines substrate binding and proteasomal cleavage, but that up to 5 aa to both sides of the proteolytic site may codetermine whether a cleavage occurs or not. While according to extrapolations from the three-dimensional structure of yeast proteasomes the exchange of LMP2 for δ renders the preference for the P1 residue from acidic to hydrophobic (54, 52), an obvious modification of the P1 pocket could not be predicted for the exchanges of LMP7 for MB-1 and MECL-1 for MC14. It would therefore be interesting to investigate whether LMP7 and MECL-1 introduce preferences for positions other than P1 and whether proline could be one of them. Finally, it is noteworthy that the 11-mer fragment that is produced in 6-fold excess by immunoproteasomes compared with constitutive proteasomes is liberated by cleavage C terminal of an arginine residue. Arginine is a prominent proteasome cleavage site and is used by several human MHC class I molecules as a C-terminal anchor residue. Interestingly, the constitutive homologue of MECL-1, named MC14 in the mouse and PUP1 in yeast, was shown to be in charge of cleavages C terminal of basic residues (55, 53, 56). It would therefore be interesting to test whether the incorporation of human MECL-1 into the proteasome may enhance the cleavages C terminal of arginine.

New and convincing evidence that the incorporation of LMP2, MECL-1, and/or LMP7 can determine the fate of an epitope have been reported while this work was in revision. Sewell et al. (57)

reported that an HLA-A0201-restricted epitope from the N terminus of HIV-1 reverse transcriptase was only presented when cells were expressing LMP7. Also, an epitope from influenza matrix protein could only be presented on HLA-A0201 when LMP7 was expressed in the infected cells (58). Sijts et al. showed that an HLA-Aw68-restricted epitope from hepatitis B virus core Ag could only be presented under IFN-γ stimulation, and in vitro results suggested that LMP2, LMP7, and MECL-1 were required for the epitope generation (59). In contrast to these studies, the generation of several tumor epitopes was recently shown to be abrogated by IFN-γ treatment of tumor cells, and at least for the RU1 Ag of renal carcinoma the overexpression of LMP2, LMP7, and MECL-1 was shown to reduce the presentation of an HLA-B51-restricted RU1 epitope (60). Taken together, it appears that although LMP2, LMP7, and MECL-1 incorporation seems to slightly increase the total amount of suitable class I ligands (15), the effect on the single epitope can be either deleterious or beneficial. It is therefore quite important to note whether a given CTL epitope was characterized in presenting cells that do or do not express LMP2, LMP7, or MECL-1.

A somewhat surprising finding of this study was the fact that overexpression of PA28α and PA28β to the same or even higher degree as obtained with IFN-γ stimulation did not alter the presentation of the NP118 epitope. Previously, we have found that either the single overexpression of PA28α (34) or the concerted expression of PA28α and β enhanced the presentation of T cell epitopes from mouse CMV pp89 protein or the NP of influenza virus. Mechanistically, the detailed analysis of in vitro digests suggested that PA28 may induce the proteasome to perform concerted dual cleavages that would liberate fragments of appropriate length for MHC class I binding (35). It was proposed that PA28 may achieve these dual cleavages by coordinating the cleavage reactions of two juxtaposed active centers of the proteasome, which can be spanned by a nonameric peptide in extended conformation. However, this effect should in theory be of advantage for the generation of all epitopes. Thus, our results suggest that PA28 functions in a different and more epitope-specific way. Although it has meanwhile been confirmed by a number of groups that PA28 markedly alters proteasomal cleavage reactions in vitro (13, 35, 36, 50), the principles and mechanisms of PA28 function still need to be better defined.

IFN-γ induces a multitude of genes that affect Ag processing and also have direct adverse effects on viral replication. It is hence not easy to decide to which degree the different gene products contribute to the elimination of LCMV in vivo. The application of neutralizing Abs to IFN-γ in mice showed that this treatment enhanced the viral load in the course of the immune response (61) and that neutralization of IFN-γ could prevent elimination of the virus (62). More recent experiments in gene-targeted mice deficient for IFN-γ showed that in the absence of IFN-γ, LCMV could be eliminated after an acute experimental infection, although the cytolytic activity of LCMV-specific CTLs from these mice was reduced by a factor of 2–3. Similarly, mice that lacked the IFN-γ receptor were able to clear an acute infection with LCMV, but virus titers on day 10 postinfection remained 10 times higher in spleen and lung, but 1,000 and 10,000 times higher in liver and ovary, respectively (63). Interestingly, the organs with the lowest titer of LCMV in IFN-$\gamma R^{o/o}$ as compared with wild-type mice (spleen, lung) were organs of high constitutive expression of LMP2, LMP7, and MECL-1 (64). Whether LMP2, LMP7, and MECL-1 contribute to these in vivo effects should now be investigated in gene-targeted mice deficient for LMP2 or LMP7.

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